EEDP-04-24
July 1995

Environmental Effects of Dredging Technical Notes

Methods for the Assessment of the Genotoxic Effects of Environmental Contaminants; Subcellular Effects

Purpose

This technical note is the first in a series of three that outline and describe the principal methods that have been developed to test the potential of environmental contaminants for causing mutagenic, carcinogenic, and teratogenic effects. This technical note describes methods used to discern genotoxic effects at the subcellular level, while the second in the series (EEDP-04-25) describes methods used to discern genotoxic effects at the cellular and organ/organism level. In the third technical note (EEDP-04-26), recent literature citations for each topic are listed to assist readers in locating source information. Technical Note EEDP-04-26 also includes a glossary of terms.

The information presented in these technical notes is intended to provide Corps of Engineers personnel with a working knowledge of the terminology and conceptual basis of genotoxicity testing. To develop an improved understanding of the concepts of genotoxicity, readers are encouraged to review "A Primer in Genotoxicity" (Jarvis, Reilly, and Lutz 1993), presented in Volume D-93-3 of the *Environmental Effects of Dredging* information exchange bulletin.

Background

Many contaminants of dredged material acting singly or in combination are toxic to exposed organisms through effects on DNA. Such effects are usually the result of low-level exposures lasting a long time. These effects can result in reproductive failure of organisms, impaired growth and development (especially of early life stages), and tumors, sometimes cancerous, in fishes and vertebrate wildlife. Collectively, such effects are called "genotoxicity" and result from damage to the genome of a cell. The damage is heritable, that is, passed on to future cell generations upon duplication of the affected cells.

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Tests are available to detect genotoxic effects at each level of organism integration: subcellular/molecular, cellular, tissue/organ, and whole organism. These tests, the most important of which are described in these technical notes, have been developed for and applied specifically to the genotoxicity of single-chemical compounds to mammalian species.

Although tests of sediment genotoxicity are not routinely applied in regulatory contexts, the potential for their requirement in special circumstances is implied by the language of public law. For example, the Marine Protection, Research, and Sanctuaries Act of 1972 (PL 92-532), which regulates disposal of dredged material in the oceans, specifically prohibits open-water disposal in other than trace amounts of "known carcinogens, mutagens, or teratogens or materials suspected to be carcinogens, mutagens, or teratogens by responsible scientific opinion." In addition, the emphasis in environmental toxicology over the last decade has increasingly shifted away from the catastrophic endpoint (death of individual organisms in acute exposures) to chronic and sublethal toxicities having the long-range ability to seriously affect the survival and well-being of populations of organisms.

For this reason, research aimed at developing a methodology for testing the genotoxic potential of contaminated sediments to aquatic organisms is being undertaken at the U.S. Army Engineer Waterways Experiment Station. This technical note, prepared under the sponsorship of the Long-term Effects of Dredging Operations (LEDO) Program, provides background information to assist potential users of genotoxicity testing methods in evaluating and interpreting test results.

Additional Information

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Effects on Genetic Material (Refs. 1-10)*

A mutation occurs when a nucleotide is chemically modified, deleted, or substituted. Certain environmental contaminants act as mutagens in that they

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* Refer to bibliographic citations 1 through 10 in *Environmental Effects of Dredging* Technical Note EEDP-04-26.

covalently bind to DNA nucleotides, chemically modifying the DNA. The cell contains DNA repair enzymes that can repair mutations under normal circumstances. However, when the organism is exposed to an excessively high level of a mutagen, the DNA repair enzymes may not be able to repair all of the mutations or may misrepair some mutations by deleting the nucleotide rather than replacing it, or by substituting a wrong nucleotide for the mutated one. Depending on the location of the mutation, the number of mutations, and whether the mutation is repaired by the cellular DNA repair enzymes, a mutation may progress to tumor formation or cancer in the organism.

Two basic types of assays for mutagenicity are available. Bacterial assays such as the Ames assay and the proprietary assay, Mutatox, are designed to detect the presence of mutagenic compounds in a sample. The other type of assay, exemplified by ^{32}P-postlabeling and the alkaline unwinding assay, is designed to determine whether a particular organism or cell has experienced mutations.

Ames Assay (Refs. 11-33)

The Ames assay uses strains of a bacterium, *Salmonella typhimurium*, that have been purposely mutated so that they cannot produce the amino acid histidine, which is required for survival. In order for colonies of these bacteria to grow, they must be cultured on media containing sufficient levels of histidine. For the assay, the bacteria are incubated with the test compound on culture media containing trace levels of histidine and are checked for colony formation. If the test compound is mutagenic, the genetically altered bacteria will reverse mutate, or revert, to the "wild type" and be able to synthesize their own histidine. Thus, bacterial colonies growing on the histidine-deficient media indicate the presence of mutagens in the growth medium. Chemicals that are mutagenic in the Ames assay are usually carcinogenic in life-cycle rodent bioassays.

Mutatox (Refs. 34-44)

Mutatox is a relatively new, easy to perform proprietary assay that uses a dark mutant of the bacterial strain *Photobacterium phosphoreum*, which normally bioluminesces (like fireflies). These dark mutants of *P. phosphoreum*, similarly to the *S. typhimurium* tester strains used in the Ames assay, revert to the wild type in the presence of mutagens. The mutation causes *P. phosphoreum* to bioluminesce, and the light produced is easily measured with a luminometer.

A characteristic of prokaryotes such as *S. typhimurium* and *P. phosphoreum* is that, unlike almost all vertebrate and invertebrate species, they do not contain cytochrome P450. Cytochrome P450 is a family of membrane-bound enzymes found primarily in liver cells that function in steroid metabolism and in the metabolism of foreign compounds. Many environmental contaminants, such as polycyclic aromatic hydrocarbons (PAHs), are not genotoxic in their original chemical form, but can be biotransformed (metabolically activated) to a reactive chemical form by cytochrome P450 enzymes. The Ames assay and

Mutatox can be used to distinguish between contaminants that require bioactivation and those that do not, by inclusion of a rat liver preparation (S9) containing cytochrome P450 with the bacteria and test extract. The Ames assay is typically performed both with and without exogenous metabolic enzymes to differentiate direct-acting mutagens from promutagens, or those that must be metabolized for activity.

^{32}P-Postlabeling (Refs. 45-50)

^{32}P-Postlabeling is a highly sensitive method used to determine whether DNA from a particular organism has been chemically modified by a mutagen, that is, has formed adducts. DNA is extracted from the organism, usually either from the liver or the whole organism, and enzymatically digested to a mixture of normal and adducted nucleotide monomers. The nucleotides are then enzymatically labeled with ^{32}P, and the adducts are enriched relative to the normal nucleotides either enzymatically or by extracting into n-butanol. Adducted nucleotides are quantitated using autoradiography, which entails laying a piece of photographic film over the TLC plate used to separate the different nucleotides and letting the gamma rays from the ^{32}P label expose the film.

Alkaline Unwinding Assay (Refs. 51-61)

Alkaline unwinding assays are used to indirectly measure adduct formation by determining the number of strand breaks that occur. The DNA from exposed cells or organisms is isolated and subjected to alkaline conditions (pH > 10.5) by the addition of a strong base. The alkalinity simultaneously causes the DNA to break at the sites of most DNA adducts and causes the DNA to unwind from its normal double-stranded configuration to the two single DNA strands. Higher numbers of DNA adducts cause higher numbers of DNA strand breaks which, in turn, causes a faster rate of double-stranded DNA unwinding as compared to normal DNA. The rate of DNA unwinding is calculated and can be expressed as number of adducts per milligram DNA.

A variation of the alkaline unwinding assay is the single cell gel (SCG) assay, also known as the "comet" assay. For the SCG assay, cells (for example, blood cells) from an exposed organism or cells grown in culture and exposed to extracts are embedded into a gel-coated microscope slide. The slide is incubated in an alkaline solution, to allow DNA unwinding, and is subjected to electrophoresis. The cells and DNA are stained and scored with a microscope. Cells with damaged DNA have the appearance of a comet, with DNA trailing from the cell body, while cells with undamaged DNA appear normal.

Unscheduled DNA Synthesis (Refs. 62-65)

Unscheduled DNA synthesis is a test for mutagenicity that monitors DNA repair following DNA damage from a mutagen. Cells (typically, isolated liver cells from an exposed organism or cells exposed in cell culture) are incubated

with ^{3}H-thymidine, a radiolabeled nucleoside. In repairing the damaged DNA, ^{3}H-thymidine is incorporated into the DNA. The amount of repair, monitored autoradiographically as in ^{32}P-postlabeling, is proportional to the amount of damage.

Nongenotoxic Effects on Adjunct Systems (Refs. 1-10)

Exposure to genotoxic agents usually affects living organisms in numerous ways other than just by causing DNA damage. The inclusion of observations on adjunct systems concurrently with genotoxicity measurements can contribute substantially to a correct interpretation of the result. For example, a chemical may produce a genotoxic effect such as significant mutation in the Ames assay, while at the same time causing either an increase or a decrease in one or more nongenotoxic parameters. It would be highly desirable to know the effects of increasing or decreasing these parameters with regard to the initiation or promotion of cancer. By understanding the roles played by adjunct systems, changes that occur in their function through exposure to genotoxicants can be interpreted in terms of potential for the development of cancer or abnormal early development.

Cytochrome P450 (Refs. 66-81)

Cytochrome P450 is a group of enzymes located primarily in metabolically active tissues (such as the liver, spleen, kidney, and lungs) that function in steroid metabolism and also metabolize xenobiotic compounds such as PAHs. Exposure to certain groups of compounds including polyhalogenated dibenzo-*p*-dioxins and furans, polychlorinated biphenyls, and PAHs induces the formation of specific cytochrome P450 enzymes that are normally not present or present in very low quantities. This enzyme induction is believed to have a promotional effect on initiated cells, in that it stimulates cells that have been predisposed, or initiated, to cancer formation to become cancerous. Induction of cytochrome P450 also leads to a higher rate of xenobiotic metabolism, which is generally beneficial to the organism. In the case of several of the PAHs, however, metabolism leads to activation of the parent PAH to a reactive metabolite, which can form adducts with DNA, causing mutations.

Knowledge of the mechanism and effects of cytochrome P450 induction can provide tools for screening sediment contamination. Monitoring cytochrome P450 levels in organisms exposed to sediments either in the laboratory or in the field can give an index (biomarker) of contaminant exposure. Cytochrome P450 induction is also the basis for a bioanalytical assay for dioxins, the EROD induction assay.

Bile Metabolites (Refs. 82-85)

Once a toxicant is absorbed into the bloodstream of an organism, it is transported through the liver where a large portion of the toxicant is absorbed into the liver cells (hepatocytes). Hepatocytes are rich in cytochrome P450 and

other metabolizing enzymes, and a portion of the absorbed toxicant is metabolized. In the case of most environmental contaminants, metabolism leads to the formation of unstable metabolites, which usually immediately react with some cellular component in close proximity to the site of metabolism. The majority of these unstable metabolites react with intracellular water, typically forming innocuous secondary metabolites. A fraction of the unstable metabolites reacts with other cellular components, including DNA (forming an adduct) and bile. Bile is the waste product formed by hepatocytes, which is secreted into the gall bladder and finally into the intestinal tract. Bile can be isolated from an organism and analyzed, usually using high performance liquid chromatography, for specific bile metabolites, giving an index of exposure (biomarker) to that particular compound.

Phase II Enzymes (Refs. 86-90)

The enzymes that catalyze the metabolism of xenobiotic compounds are generally classified into two groups, phase I enzymes and phase II enzymes. Phase I enzymes, which include cytochrome P450 monooxygenases (or mixed function oxidases, MFOs), act to expose or add functional groups on the parent xenobiotic molecule, increasing its water solubility. Phase I metabolites often are excreted in the urine. Once a compound undergoes phase I metabolism, or if the parent molecule contains a phase I functional group, the molecule may undergo phase II metabolism, as illustrated in Figure 1. Phase II metabolism adds, or conjugates, a bulky endogenous molecule to the parent molecule. Endogenous molecules that are conjugated in phase II metabolism include glucuronic acid (a glucose derivative), amino acids, sulfate groups, methyl groups, glutathione, and acetyl groups. Phase II metabolism generally facilitates biliary as well as urinary excretion.

Phase II metabolism acts as a protective mechanism for the cell, in that reactive phase I metabolites have an alternative site to bind rather than to DNA. A compromise in phase II metabolism may lead to genotoxic effects. Phase II enzyme systems are saturable; that is, they are overwhelmed when presented

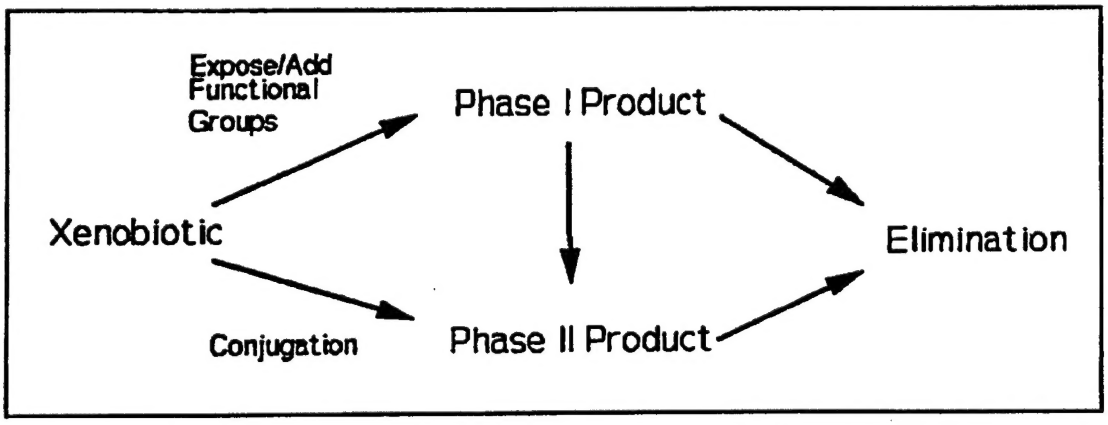

Figure 1. Schematic of phase I and II metabolism

with excess xenobiotic or phase I product and cannot metabolize the reactive compounds fast enough to prevent DNA damage. The endogenous phase II conjugating molecule may also be depleted.

Heat Shock Proteins (Refs. 91-93)

Heat shock proteins (HSP) are a family of proteins found in the cytosol of almost all types of cells. Levels of HSP are increased upon exposure to elevated temperatures and other cellular stresses. Little is known of the function of HSP, but it is generally believed that these proteins enable cells to have an increased thermotolerance, help stabilize other proteins, and aid in translocation of macromolecules within the cell. Elevated levels of HSP have been suggested as a biomarker of exposure to environmental contaminants. HSPs are detected using electrophoresis or column chromatography.

Cytotoxicity (Refs. 94-100)

The effect of DNA damage depends on the function of the area of the DNA to which the damage occurred and whether the damage was repaired. Damage to nonsense regions of DNA (regions that do not code for a particular protein) will usually not have an effect on the function of the cell, but may alter replication of DNA during cell division. In this case, daughter cells may not be formed, leading to the death of the parent cell. Damage to genes that code for functional proteins (enzymes) or structural proteins necessary for cell viability would also cause cell death. Since DNA damage occurs in a seemingly random manner to cells within an organ, not all of the cells with DNA damage would be expected to die. Some cells with DNA damage may be initiated while others die. Thus, cytotoxicity (cellular injury or death) may be used as an indirect determinant of genotoxicant exposure.

Cytotoxicity may be evaluated in several ways. Upon cell damage or death, most cells release enzymes or other proteins that can be used as markers of cytotoxicity. Alanine aminotransferase (ALT) is such an enzyme that is indicative of in vivo liver cell (hepatocyte) injury or death. Serum levels of ALT increase dramatically upon low-level hepatotoxicant exposure. In vitro cytotoxicity may be measured with dyes such as trypan blue, neutral red, ethidium homodimer-1, and calcein AM.

Ornithine Decarboxylase (Refs. 101-109)

Ornithine decarboxylase (ODC) is an enzyme indicative of cellular proliferation, signaling possible exposure to a cancer promoter. ODC removes a carboxyl ($-CO_2H$) group from ornithine, a derivative of the amino acid arginine, to form putrescine, the initial product in the polyamine biosynthetic pathway. Polyamines are normally present at very low levels in quiescent cells, but are elevated many-fold during periods of active cell division. To assay for ODC activity, livers from exposed organisms are isolated and prepared using ultracentrifugation. Ornithine having a radiolabeled carboxyl group is

incubated with the enzyme preparation, and the metabolized radiolabeled carboxyl group, which is liberated as a gas, is trapped and quantitated.

Oxidative Stress (Refs. 110-118)

While oxygen (O_2) is essential for all multicellular organisms, some forms of oxygen produced during the metabolism of oxygen, that is, superoxide anion radicals ($O_2^{\cdot -}$), hydroxyl radicals (OH·), and hydrogen peroxide (H_2O_2), are highly reactive. These oxyradicals can upset the reduction-oxidation (redox) potential of the cell, leading to a highly oxidizing environment within the cell. The redox potential of cells is normally tightly controlled by cellular antioxidant defense mechanisms. These mechanisms include the enzymes superoxide dismutase, catalase, and glutathione peroxidase, as well as vitamins (β-carotene, α-tocopherol, retinoic acid, and ascorbic acid), and the tripeptide glutathione. Cellular antioxidant defense mechanisms can be overwhelmed by xenobiotic chemicals that induce oxidative stress, exemplified by paraquat and quinones. Consequently, genomic function can be impaired due to alterations in DNA, such as the formation of 8-hydroxydeoxyguanosine adducts. Enzyme function may also be inactivated and cell membranes may be disrupted due to protein and lipid oxidation, ultimately resulting in cell death.

Oxidative stress is evaluated by measuring either the oxygen radicals themselves, oxyadducts such as 8-hydroxydeoxyguanosine, or the induction of oxidative stress defense mechanisms such as glutathione.

INTERNET DOCUMENT INFORMATION FORM

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D. Currently Applicable Classification Level: Unclassified

E. Distribution Statement A: Approved for Public Release

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